Reference: *Biol. Bull.*, **155**: 627–639. (December, 1978)

COMPARISON OF THE GRADIENT OF SETAL DEVELOPMENT OF UROPODS AND OF SCAPHOGNATHITES IN *ASTACUS LEPTODACTYLUS*[1]

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The intermolt stages of decapod crustaceans are usually defined according to the criteria of Drach (1939; 1944). Many authors subsequently adapted this method to their own material for treating diverse physiological problems. Drach and Tchernigovtzeff (1967) then generalized this technique for application to all crustaceans. The original method allowed rigorous classification, but required injury to the animal due to the extraction of appendages used for microscopic observation. In order to choose from the tremendous number of crayfish available in populations, it was necessary to find criteria for stages within the cycle that would allow a quick and simple identification of molt stages without damage.

The observation of more easily accessible appendages was suggested by several authors: the pleopods of the macruran, *Homarus americanus,* (Aiken, 1973) or of some Stomatopoda (Reaka, 1975); the antenna scales of a natantian, *Palaemon paucidens,* (Kamiguchi, 1968); the uropods of three natantians (Scheer, 1960); or appendages of an anomouran, *Petrolisthes cinctipes* (Kurup, 1964); or of the macrurans, *Homarus americanus,* (Donahue, 1954; Rao, Fingerman and Fingerman, 1973); *Orconectes sanborni* (Stevenson, Guckert and Cohen, 1968) and *Parastacoides tasmanicus* (Mills and Lake, 1975).

The need to keep the animals uninjured together with the fact that they possess neither bladed pleopods nor antenna scales, brought us to use the uropodal blades in crayfish to determine molting stages as well as to predict ecdysis. The data obtained from uropods is set out, and the validity of our method has been tested by a comparative study of the scaphognathites.

Materials and Methods

Hardness of the shell

The hardness of different parts of the cephalothorax and the appendages can be estimated by palpation following the classical sequence set out by Drach (1939).

Examination of the uropods

After apolysis, two methods can be used to predict the duration of premolt stages. The first, worked out on *Jasus paulensis* (Vranckx, in preparation), measures the retraction of epidermis which has pulled away from the old cuticle.

[1] This paper is dedicated in homage to Professor Pierre Drach on his jubilee, and the authors also wish to thank him for critical review of this manuscript.

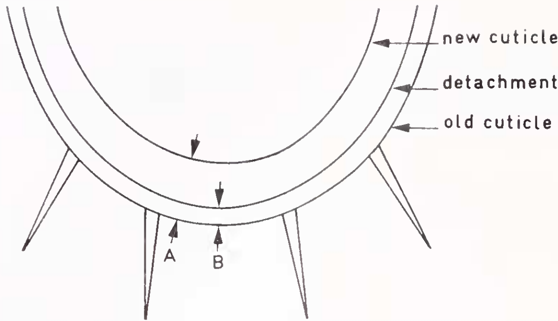

FIGURE 1. Edge of crayfish uropod during the premolt stages showing the measurement of retraction rate index R of the epidermis, which has pulled away from the old cuticle. For a definition of "R", see text.

It is measured with an ocular micrometer, and the retraction rate index R is calculated: R = A/B = distance from external edge of the old cuticle to external edge of the new one/thickness of old cuticle (Fig. 1). To make the measurement easy and to standardize this method, the reading is made on the external edge of the uropod. Care must be taken that detachment of the epidermis, if any, is really related to the premolt state and not to any adjacent injury.

The second method follows the setal evolution in the uropod blades and can be studied with a stereoscopic microscope (Wild × 50). The moistened uropod is set on a rough glass and viewed as a transparency. This determination in *Astacus leptodactylus* is more difficult than in other macrurans because of the thickness and opacity of the uropod blade and its double-bordered setal range (primary and secondary). The internal part of the exopodite of the uropod is examined below the dieresis line between the distal and proximal parts. This thin part of the uropod does not possess the double set of setae.

Comparative study of scaphognathites

Pieces of scaphognathite on both sides of the muscular insertion are picked off and mounted in water with the forepart uppermost. Similarly, the endites of the second maxilla are observed with a light microscope.

RESULTS

Hardness of the shell

The post-molt cuticle that was very soft in stage A gradually becomes harder and harder until the stage C2. In addition, more information can be obtained by feeling the abdomen above the pleurite attachments: it remains flexible crosswise until the end of stage C1.

The shell is hard from C2 through C3 to stage D2, but a certain flexibility can sometimes be noticed from later stage D1 in the branchiostegites within the area underneath the molting line, although this is clearly distinct from stage D3. Then, from stage D3 to ecdysis, the exuvial lines of the shell soften.

It should be noted that after molting the subjects are often brightly colored, tending toward blue throughout stage C3. During this stage the shell is brittle and does not break evenly as in stage C4, when the membraneous layer was being formed. As the animals reach stage C4, the color of the cuticle becomes greenish or brownish, then deepens and turns to a dull color.

Examination of the uropods

Within the uropods, the lacunary circulatory system stays unstructured throughout the cycle, but takes on the appearance of well-structured canals in stage A. These uropods display distinct setal matrices (Fig. 2a). They become homogeneous and opaque up to the end of stage C3. It then becomes impossible to distinguish the perpendicular ribs bordering the uropods in the setae extensions. The position and the structure of these ribs suggest that these can later become matrices or their precursors. Circulating blood cells are not very numerous.

During stage C4, the uropod is more transparent and the thickness of the cuticle is obvious. Furthermore, this stage can be at times subdivided by means of the appearance and development of a light zone that is colorless in the part below the cuticle and whose thickness is similar to the epithelium. Toward the end of stage C4, this zone is bordered by chromatophores. This could indeed represent a transformation of the epithelium. Blood cells are more or less numerous and move about freely throughout the uropods.

In stage DO, the homogeneous aspect disappears, and in compressed uropods the inner fibrillar structures situated in the setae extensions are visible. These formations do not extend beyond one quarter of the distance (D) from the cuticle edge to the first external spine of the dieresis line. They appear like two parallel lines and must correspond to the future matrix (Fig. 2b). To determine stage DO, it is more convenient to seek this very consistent trait, rather than the retraction that is difficult to observe because of the uropod's thickness. Furthermore, especially during stage DO but also sometimes in stage D1′, the hemocytes are less easily transported by the circulation, and they seem to stick to these fibrillar structures more than in other stages.

In stage D1′ the same fibrillar structures are extended and reach half the distance (D) from the shell edge to the first spine. On the other hand, no split can be seen within the thickness of these structures (Fig. 2c).

Due to the splitting of the epidermal tissue, these structures clearly appear in stage D1″ as invaginated setae which extend beyond three quarters of the edge–spine distance (D), and remain clear. On and after this stage where retraction is always obvious, the edge of the new uropod is lightly scalloped (Fig. 2d). Hemocytes are numerous, and they move about. During stage D1‴ the setae reach the bottom of their own invaginations at the distance (D) we previously defined but the setae remain clear, just like the surrounding tissue (Fig. 2e). At times barbules are noticeable in the more transparent parts. Blood cells no longer stick to the matrix or more exactly to the now invaginated seta. The opacity as well as the darkness of the invaginated setae in relation to the surrounding tissue differentiate stage D2 from stage D1‴ (Fig. 2f).

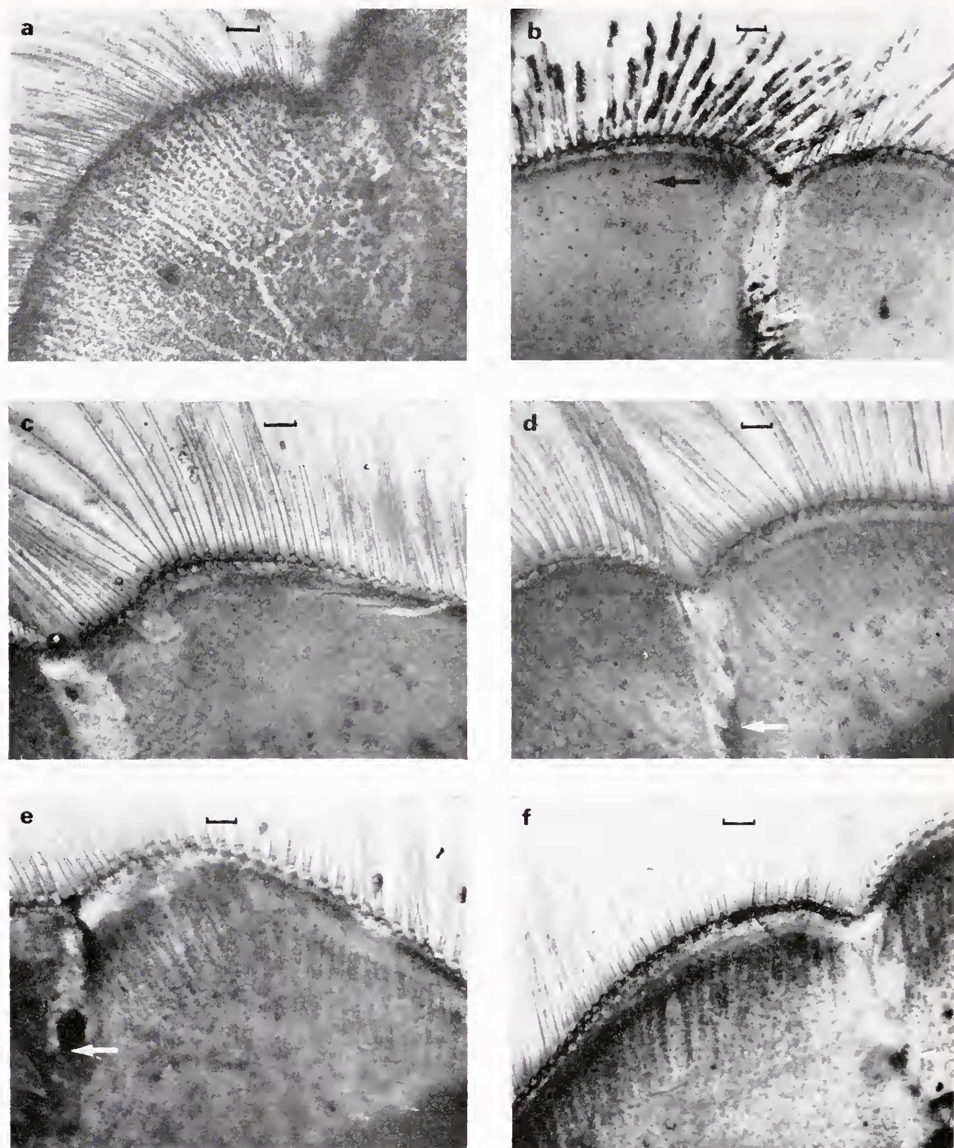

FIGURE 2. Uropod exopodites from *Astacus leptodactylus* examined with a stereoscopic microscope: a, lacunary circulatory system taking on appearence of structured canals in stage A; b, stage DO showing the matrix indicated by the black arrow and the first spine indicated by the white arrow; c, d and e, stages D1′ D1″ and D‴ showing the invaginated setae that progressively extend from the shell edge to the first spine; and f, stage D2 showing opacity and darkness of the invaginated setae. All scale bars equal 0.2 mm.

In stage D3, folds in the underlying integument can be noticed, as Aiken (1973) reported in the lobster. They must not be confused with the scalloped designs formed in the old cuticle. Moreover, there are fewer circulating cells.

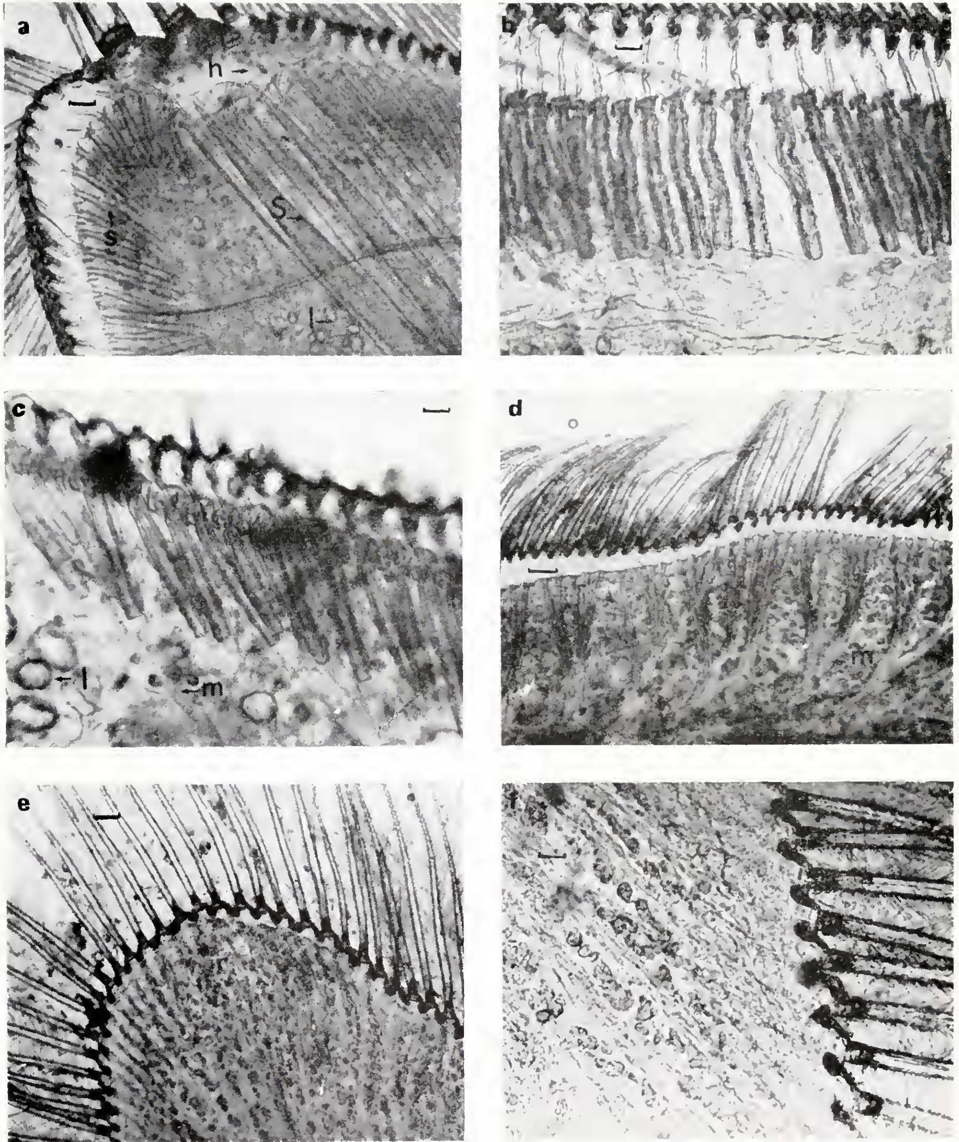

FIGURE 3. Scaphognathites examined with a light microscope (sizes of scale bars in parentheses) : a, epipodite in stage D1‴ (150 μ) ; b, setae with single matrix (60 μ) ; c and d, setae with compound or connected matrices (75 μ and 200 μ) ; e and f, stage DO showing matrix swelling (200 μ and 50 μ). Abbreviations are: S, large setae; s, small setae; h, hemocytes; l, "adipocyte" type cells; and m, matrix.

It should be noted that from stage D1 on, when retraction is substantial, the blood cells are often visible in the exuvial space.

Furthermore, the retraction index, showing a progressive retraction from stage DO to stage D4 in *Jasus paulensis* uropods and thus forecasting the date

of ecdysis (Vranckx, in preparation), cannot be applied to *Astacus leptodactylus* because of the thickness of the ends of the uropodal blades. In contrast, it can be applied to other Astacidae such as *Procambarus clarkii* (unpublished results).

Study of scaphognathites

From a morphological point of view, the setae bordering the scaphognathite are not altogether similar. Actually, three kinds should be distinguished. First a small number of large setae, situated at the apex of the epipodite, show their matrices (Fig. 3a). In the second kind also, matrices separated from each other at their bases are shown in some small setae (Fig. 3b). Both kinds of setae belong to the type that we call "single matriced". In the third kind the matrices of three to five small setae are clustered together at their bases and are called "compound or connected matrices" (Figs. 3c and 3d).

Even though all setae have synchronous development, examination of the epipodite's two or three biggest ones shows, interestingly enough, that they always seem to be one stage ahead of the others during the D1 phase. The relative discrepancy of staging between biggest and smallest setae is possibly due to the fact that the size of the former renders any phenomenon more obvious (appearance of the fold in stage D1′, barbules). The study of these big setae can be important in clearing up any doubtful cases.

Contrary to the observations made from uropods in which movements of the matrix are seen (*i.e.,* individualization of the matrix from the surrounding tissue or retraction of the Aiken's setal organ) it was impossible to discern any such movement in either of these single or compound matrices within the tissues. From the beginning of postmolt or, in any case, from stage C1 through C2, since this is the first stage studied in a suffcient number of scaphognathites, the matrices or their supports have been set in place. From DO, they swell and thus reduce the intermatrix space (Figs. 3e; 3f).

On the small setae, stage D1″ (Fig. 4b) appears like an intermediate stage of development which happens to be between the stage D1′, the beginning of invagination, and the stage D1‴, in which each invaginated seta attains its greatest depth and has quite obvious barbules (Fig. 3a). In addition, small refractile nodes are seen in D1‴ inside the setae next to the barbule inplantation areas. On the large setae, the barbules can already be distinguished, even though the invagination is not yet finished (*i.e.,* as soon as early D1″). Stage D1‴ can be distinguished from stage D2 not only by means of its more or less strong tissue opacity, as on the uropods, but also by the refringence of the epithelium between the setae (Fig. 3c). Throughout stage D2 to D3 the setae are very clear, but they do not quite reach the bottom of their matrices (Figs. 3c; 3d).

"Adipocyte" type cells, likely the cells of Sewell (1955) are observed inside the scaphognathite. Their size and localization vary during the cycle. These often well developed cells, which reach the edge of the epithelium in stage C1–C2, seem to regress in C3 and C4, and remain thus until the stage DO. They then increase in number in the central part of the scaphognathite, although they remain small. They grow in size until the end of D1‴ or D2, when they become extremely fragile (Figs. 3a, 3c; 4e). From this latter stage until the ecdysis they have been seen disrupting, and they disappear under microscopic observation.

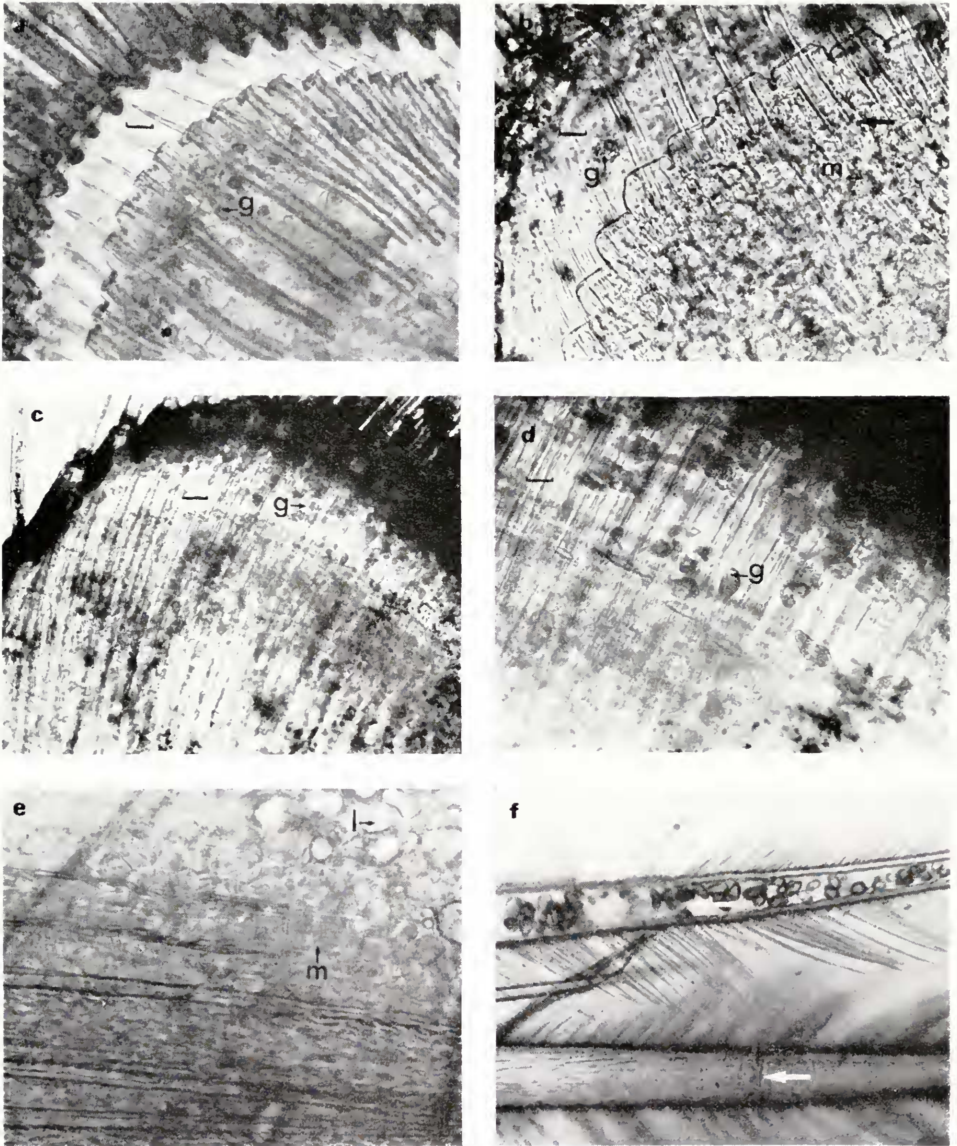

FIGURE 4. Scaphognathites observed with an optical microscope (sizes of scale bars in parentheses): a, scaphognathite in stage D2 (g, represent the granulocytes) (75 μ); b, scaphognathite in stage D1′ (the arrow shows the invagination and m, represents the matrix) (30 μ); c and d, endites from second maxilla, with granulocytes in the exuvial space (100 μ and 30 μ); e, large setae of epipodite and its matrix (shows the "adipocyte" type cells and m represents the matrix); and f, setae with the bottom of invagination fold indicated by the arrow and cellular components unretracted.

The origin of the differences in the "adipocyte" type cell localization is perhaps due to the variations of physiology of each animal studied and to the stages of development of the matrices.

The number of circulating hemocytes varies during the molt cycle. There is an increase of the blood cell density, especially that of granulocytes, in C2–C3, then in D1‴–D2 (Fig. 4a). Moreover, granulocyte accumulations appear between the former cuticle and the new one (Fig. 4b); this phenomenon is especially clear at the end of stage D1, when the maxillary endites are examined (Figs. 4c and 4d).

Durations of the premolt stages

The length of the premolt stages was not precisely evaluated. It can nevertheless be estimated from 4 to 8 weeks, starting from middle DO when proecdysis has already begun. This time bracket is quite long because some animals that are labelled D1′ occasionally molt in the aquarium prior to those labelled D1″ observed at the same time. Although these latter results appear illogical, they may be due to factors in the animals' past lives before we obtained them (injury, environmental factors).

Discussion

The duration of the premolt stages is greater than that indicated by Stevenson *et al.* (1968) for *Orconectes sanborni,* but under different experimental conditions: their animals were much smaller and the water temperature much higher. Mills and Lake (1975) estimated the premolt period at 14 weeks from stage DO, using southern hemiphere, autumn animals. The fact that the molting cycle unfolds with slight differences according to the season may explain their findings. Indeed, in *Astacus leptodactylus* the spring and summer crayfish shift imperceptibly from stage C4 to premolt, and in certain subjects that molt twice during the summer, the stage C4 is reduced to the bare minimum so that C3 tends to go almost directly into stage DO. On the other hand, some autumn animals seem to stop their molt cycle at the beginning of stage DO and not in stage C4, as Aiken (1973) clearly observed in *Homarus americanus,* and Vranckx (unpublished results) also observed in *Jasus paulensis.* For the postmolt animals our findings agree with those of Stevenson (1968).

The division of the intermolt cycle into important substages (Drach, 1939) is mainly determined by morphological characteristics. Drach defined stage D as the premolt period and subdivided it into four substages (D1 to D4).

After further research, another stage, DO, was introduced into the stage D (Charniaux Legrand, 1952), and described by Drach and Tchernigovtzeff (1967) as "the retraction of epidermis and setal matrices." These authors claim this phenomenon is the first morphologically obvious feature showing that the animals have entered the premolt stage. Further studies about the molt physiology have shown obvious variations in results for stage DO, while relatively homogenous data were obtained for the other stages. Growth studies (Aiken, 1973, on *Homarus;* and Vranckx, unpublished results, on *Jasus* and *Astacus*) have shown different kinds of growth curves as the animals enter the premolt phase. These growth curves differ according to the investigated animals and the season in which they enter the premolt. The shape variations of these curves are especially related to kinetics of the DO phase. At this stage, too, the growth

rate of the regenerating limb bud varies in *Gecarcinus lateralis* (Tchernigovtzeff, 1972). The great variability of epidermal DNA content in stage DO compared to other premolt stages in *Orconectes sanborni* (Stevenson, 1972) is another fact that could support the idea of a heterogenous stage DO. The heterogeneity of stage DO thus described seems well established, for Drach and Adelung's specifications do not really coincide for this stage (Spindler, Adelung and Tchernigovtzeff, 1974).

This difficulty has brought different authors to subdivide stage DO into two or three substages ("early DO", "middle DO" and "later DO") to explain their results. Once this stage is subdivided, it seems that gastroliths are never found at the beginning of DO in *Astacus leptodactylus* (Vranckx and Durliat, unpublished results); furthermore, Tchernigovtzeff (1965) showed that the mitotic rise was seen in middle or later DO in *Carcinus maenas.*

From all these data, it seems that different physiological phases can be included in a single morphological stage, DO. A first period is characterized by the retraction of the epidermis (beginning of DO, according to several authors) which does not indicate the beginning of premolt, but only the end of integumentary quiescence. So, Skinner (1962) noticed minimal respiratory activity (QO_2) in crustaceans during stage DO. This raises the problem of whether anecdysis, if any, is found in stage C4 or rather in early DO. Anecdysis in stage DO would explain the precocious detachment of the nail in *Orchestia* (Charniaux-Legrand, 1952) as well as the blocking of certain *Carcinus maenas* by *Sacculina* parasites in C4–DO or DO (Andrieux and Berreur, unpublished results). This may explain why Keller and Adelung's (1970) histological figures of DO in *Orconectes limosus* resemble those of C4. Thus, at the beginning of stage DO, the morphological state (apolysis) is not identified with a physiological state, since detachment is not necessarily followed by ecdysis (Jenkin and Hinton, 1966).

On the other hand, a second phase in DO is characterized by a mitotic rise in the epidermis when the animals truly re-enter the premolt phase. Furthermore, several authors have investigated the action of the ecdysteroids on the onset of the molting activity and our interpretation could explain their data on a new basis. So, Willig and Keller (1973) have evidenced by bioassay that, although in DO characterized by apolysis, 75% of the crayfish do not possess detectable molting hormone, they do not detect ecdysteroids in 50% of animals in stage D1! The effect of ecdysteroids on the molt has been extensively discussed by Rao *et al.* (1973) and even when apolysis is triggered off in some animals by remaining ecdysteroids, for instance the small growth observed following basal growth of the regenerating limb bud in *Sesarma* (Passano and Jyssum, 1963), it is strange that the subject becomes blocked only at the beginning of DO and does not develop further on, with the exception of Stomatopoda (Reaka, 1975). But Gilgan and Farquharson (1977) evidenced that, at a physiological level, it is not only the amount of ecdysteroids that allow the premolt process to proceed further on, but also the tissue sensitivity to the ecdysterone, which depends on the investigated stage and is significantly higher in stage D1′ than in stage C4 or DO. This stage sensitivity is perhaps related to the action of both molting and molt-inhibiting hormones. On the other hand, the fact that in autumn the molt process of our animals does not proceed further than stage DO can not be explained by a reduced

sensitivity to ecdysterone at reduced temperature (Gilgan and Burns, 1977), because our animals were kept in a tank at constant temperature. Ecological factors must then intervene in order to re-establish the premolt (Kracht, 1976). In any case, for the physiologist, it may be wiser to consider the morphological stage of DO as a terminal stage of C4. Once activation is obtained, the molting process seems irreversible after the end of DO or the beginning of D1.

When premolt begins, the hemocytes accumulate in the hypodermis and stick to the matrices. They must carry substances needed for the elaboration of the new cuticle and may be even the "activators" for epidermal proliferation, as demonstrated by Bohn (1977) in the cockroach, *Leucophaea maderae*. These cells within the hypodermis should not be confused with the precursors of lipid cells described by Sewell (1955).

Moreover, the passage of blood cells into the exuvial space as observed in insects by Barra (1969), Devauchelle (1971) and Zacharuk (1972) and perhaps noted in the lobster by Hepper (1965), probably happens during apolysis. The hemocytes might contribute to the enzymatic activity of the exuvial liquid.

The setogenesis observed *in vivo* in the scaphognathite of *Astacus leptodactylus* then occurs as Stevenson *et al.* (1968) described, in a "cylindrical zone with lower cell density", whereas in the uropods it is merely observable from middle DO on as a span of cytoplasm that reaches from the top of the future seta "into a cluster of nuclei" in the integument, which correlates with Thomas's observations (1970) in *Austropotamobius pallipes*. Hence, setae organize themselves within an epithelial cylinder (the matrix) according to the pattern described by Tchernigovtzeff (1976), which, incidently, explains Reaka's "schizocoelous slits" (1975). There are not two types of setogenesis as Mills and Lake (1975) claim, but two kinds of quiescent matrices, depending on the species investigated. The matrix is observed either as a "setal organ" at the basis of a seta (contracted form of the matrix when it does not retract out of the lumen of the seta during the intermolt period, *i.e.*, big setae of *Nephrops'* scaphognathite, unpublished result) or as an epithelial cylinder inside the tissue (elongated form of the matrix which is due to its retraction out of the seta, *i.e.*, big setae of *Astacus* scaphognathite). Kurup (1964) and Aiken's (1973) setal organ therefore would not be a special organ, but would simply be due to the preception of nuclei and cell bodies of the matrix which leave the epithelium level and move inwards into the hypodermal tissue during the growth of the seta (Fig. 4e).

During ecdysis, setae evaginate due to the internal pressure of the animal, whereas during metecdysis, the matrices are more or less promptly retracted according to the animal, in a spring-back effect. Thus, the localization of the post-exuvial cuticular secretion of cones, which should not be confused with the trace of the invagination fold, is explained (Fig. 4f). It is true that little discrepancies are obvious, in staging *in vivo,* according to the observation of the different appendages or part of the same one (difference between large and small setae of the scaphognathite). Yet, a conclusion would be premature, since the actual deposit of the different cuticle layers is only revealed by ultrastructural studies. So the epicuticle is already present on the setae in D1′, although it is only visible in D1‴ with an optical microscope (Tchernigovtzeff, personal communication). Unfortunately, Kummel, Claassen and Keller (1970) and Chassard-

Bouchaud and Hubert (1973) do not indicate the exact chronology of the different layers of cuticle.

It is necessary to be very circumspect relative to comparative tables which suggest that the different integumental pre-exuvial layers do not appear at the same stage within the investigated species. The observation of uropods is consequently useful in defining the physiological molt stages, since it establishes the stage DO in *Astacus leptodactylus* after the premolt activation. Moreover, it is especially important for blood studies not to disturb the animal, since a single withdrawal of a small amount of hemolymph induces modification of both the number and type of hemocytes.

Summary

1. Characterization of the different stages of the molt cycle of *Astacus leptodactylus* has been established using morphological criteria of the setae observed through the uropod. This method is paraticularly useful for the scientist who wants to get replicable, physiological data without disturbing the animals.

2. Stages determined from uropod studies agree with those obtained from comparative studies of scaphognathites.

3. According to the seasons, a more or less quiet intermolt phase is observed. The animals either directly enter the premolt phase or are blocked in stage C4 or early DO. Therefore, the morphological stage DO seems to be heterogenous, and apolysis is not necessary to the onset of premolt.

4. A single mode of setal formation seems evidenced. Moreover, setae with compound or connected matrices are seen in the scaphognathite.

5. Number and mobility of blood cells in the uropods are related to the investigated stage. During the premolt stages hemocytes, especially granulocytes, are observed within the exuvial space.

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